Binding of neuronal ELAV-like proteins to the uridine-rich sequence in the 3′-untranslated region of tumor necrosis factor-α messenger RNA

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Abstract Tumor necrosis factor-α (TNF-α) is a proinflammatory cytokine that is involved in the pathogenesis of several human CNS disorders. The AU-rich element (ARE) in the 3′-untranslated region (UTR) of TNF-α mRNA is implicated in post-transcriptional control of TNF-α. In this study, we showed that a human neuronal ELAV-like protein binds to the ARE in the 3′-UTR of TNF-α mRNA. The protein binds to the uridine stretch in AUUUA pentanucleotides inside the ARE in the 3′-UTR of TNF-α mRNA. The TNF-α mRNA-binding region in the protein appears to be identical to the c-myc and IL-3 mRNA-binding regions. Moreover, this study showed that in vitro treatment of neuroblastoma cells with interleukin-4 (IL-4), which inhibits TNF-α production, reduced the expression of the neuronal ELAV-like proteins. These results suggest that the expression of neuronal ELAV-like proteins may be closely associated with the expression of TNF-α in neuronal cells.

Key words: ELAV-like protein; Tumor necrosis factor-α; RNA-binding protein; Interleukin-4; Neuron

1. Introduction

Tumor necrosis factor-alpha (TNF-α) is one of the key proinflammatory cytokines, which mediate many inflammatory processes. It has been implicated in the pathogenesis of several human central nervous system (CNS) disorders including multiple sclerosis [1,2], AIDS dementia [3,4], and cerebral malaria [5]. TNF-α is critically important in the early events leading to the initiation of experimental autoimmune encephalomyelitis. It damages oligodendrocytes and myelin, and induces apoptosis in neuronal cells via a pathway that involves the formation of reactive oxygen intermediates. Furthermore, TNF-α may also be a neurotoxin induced by human immunodeficiency virus type 1. Therefore, TNF-α appears to play a variety of significant roles in pathophysiological conditions of the CNS.

The production of TNF-α has been demonstrated in the CNS. Although microglial and astroglial cells mainly produce the TNF-α in the CNS [6], evidence has been presented that neurons synthesize TNF-α de novo [7–10]. Immunohistochemical studies identified TNF-α-like immunoreactive neurons in the hypothalamus, the bed nucleus of the stria terminalis, the caudal raphe nuclei, and along the ventral pontine and medullary surfaces [9]. In response to the systemic administration of lipopolysaccharide (LPS), TNF-α mRNA appeared in a cascade of neurons in the pericircumventricular nuclei [10]. Treating mature human primary neurons with soluble HTLV-1 Tax1 induced the TNF-α gene in the neurons [7]. TNF-α mRNA has also been detected in neuroblastoma cell lines, and stimulation with LPS/beta/gamma-interferon (IFN-γ) can induce TNF-α in neuroblastoma cells [11]. Therefore, neurons and neuron-derived cells are thought to produce TNF-α.

In many cells, post-transcriptional regulation plays a major role in the expression of TNF-α. The 3′-untranslated region (UTR) of TNF-α mRNA contains sequences that suppress the translation of TNF-α, and sequences in the 3′-UTR confer translational control of TNF-α expression. The AU-rich element (ARE) located in the 3′-UTR includes a major class of cis-elements that regulate the translation of TNF-α. This regulatory process involves the binding of cytoplasmic and nuclear RNA-binding proteins to a region or regions of mRNA, thereby increasing the stability of the mRNA. Identifying the proteins that bind to TNF-α mRNA is an important step in clarifying the mechanisms of post-transcriptional control of TNF-α expression, and much effort has already been devoted to identifying these proteins. In this study, we showed that a neuronal protein could bind to the ARE of TNF-α mRNA.

2. Materials and methods

2.1. Cell extracts

Human brain cell extract was prepared from the hippocampus of a brain, removed at autopsy from a male patient who died from a non-neurological disease. Brain sections were homogenized in phosphate buffered saline (PBS) and washed three times in PBS. After centrifugation, the pellet was resuspended in a cytosolic extract buffer (10 mM HEPES, pH 7.5, 40 mM KCl, 3 mM $MgCl_2$, 2 mM DTT, 5% glycerol, 0.5% NP-40 and proteinase inhibitors), and incubated on ice for 30 min. The nuclei were removed by two 5-min centrifugations at $5000 \times g$. The supernatant was used as the cell extract. Neuroblastoma cell extract was prepared in the same manner from 10^6 neuroblastoma line IMR-32 cells.

2.2. Synthetic ribonucleotides

Poly(U) and poly(A) synthetic ribonucleotide homopolymers were purchased from Pharmacia. The oligoribonucleotides that correspond to the ARE in the 3′-UTR of TNF-α (CACUUAUUAUUUAUUA-UUUAUUU-AUUAUUU) and c-myc (UAACAGAUUUGUAUU-UAAGAAUU-GUUUUUA) mRNA were synthesized, and their purity was confirmed by high-pressure liquid chromatography.

2.3. Electrophoretic mobility gel-shift assay

Two μg of brain cell or neuroblastoma cell extract or the indicated amount of HuC/ple21 recombinant protein [12] were incubated with 40 000 cpm radiolabeled RNA in a final volume of 20 μl in binding buffer (10 mM Tris-HCl, pH 7.5, 100 mM KCl, 5 mM $MgCl_2$, 0.5% Triton X-100, 1 mM dithiothreitol, 10% glycerol, 250 μg/ml yeast tRNA, 0.5 mg/ml bovine serum albumin) in the presence or absence of total IgG containing ANNA-1/anti-Hu antibodies or IgG from a

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healthy individual at 37°C for 10 min. After incubation, the reactions were resolved on a 6% polyacrylamide-0.5×TBE gel containing 10% glycerol. Subsequently, the gels were dried with heat under vacuum, and exposed to X-ray film for autoradiography for 2 h at −80°C. For competitive analyses, homopolymers or c-myc oligoribonucleotide were added to the reaction and resolved.

2.4. RNA UV cross-linking assays

Fifteen μg of brain cell or neuroblastoma cell extract was incubated with 40 000 cpm radiolabeled RNA in a final volume of 20 μl in binding buffer at 37°C for 10 min. The reactions were exposed to 0.12 J/cm^2 in a UV cross-linker, and were separated on 13% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE). The gels were dried and exposed to X-ray film.

2.5. Quantifying binding affinity

The binding affinity of TNF-α mRNA-HuC/ple21 complex was quantified using the gel electrophoresis techniques of Fried and Crothers [13]. HuC/ple21 was incubated with ^{32}P-labeled TNF-α mRNA for 10 min at 37°C in binding buffer. Increasing concentrations of ^{32}P-labeled TNF-α mRNA and constant concentrations of unlabeled TNF-α mRNA (1.5 μM) and the protein (2 μg) were added to the reactions. The bound RNA was separated from unbound RNA using the gel-shift assay. The dried gels were scanned and the results quantified using a phosphor screen image analyzer. The data were plotted according to the first-order rate equation given in Riggs et al. [14], and the apparent dissociation constant (K_d) was calculated by linear regression.

2.6. Treatment of neuroblastoma cells with cytokines

IMR-32 neuroblastoma cells (2×10^5/ml) were incubated with INF-γ (10 ng/ml) and LPS (1 μg/ml) for 2 h, or with IL-4 (10 ng/ml) for 2 h or the time indicated. After incubation, the cells were harvested and cell extracts were isolated. These cell extracts were separated on 13% SDS-PAGE. Proteins were transferred to nitrocellulose in a transfer electrophoresis unit at 400 mA at 4°C for 2 h. After the blots were washed, they were reacted with 1:500 diluted ANNA-1/anti-Hu antisera for 1 h, followed by a 2-h incubation with a 1:2000 dilution of alkaline phosphatase-conjugated goat anti-human IgG. The blots were developed with nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate toluidine salt.

3. Results

3.1. Binding to the ARE of TNF-α of cell extracts from human brain tissue and neuroblastoma line cells

The 3′-AU-rich sequence of human TNF-α mRNA has previously been shown to play a major role in post-transcriptional regulation. To determine the factors that are involved in the post-transcriptional regulation of TNF-α mRNA in the CNS, we examined the binding of neuronal cytosolic proteins to the ARE in the 3′-UTR of TNF-α mRNA. Cytosolic extracts were prepared from human hippocampus tissue and neuroblastoma cell line IMR-32, which is known to display many features of both cholinergic and adrenergic neurons. Previous studies by Hel et al. [15] revealed that one of the protein-binding regions of TNF-α mRNA is positioned between nt 1291 and 1320 inside the ARE, and that this region is crucial for both translational repression and LPS inducibility of TNF-α in monocytes and macrophages. Therefore, we prepared a 30-mer TNF-α mRNA oligoribonucleotide (nt 1291 and 1320) and used it as a probe for further experiments.

UV cross-linking assays revealed that TNF-α mRNA bound to cytosolic proteins in both the human hippocampal tissue and neuroblastoma line cell extracts (Fig. 1a). A single protein-RNA complex was detected in the neuroblastoma cell extract with a relative molecular mass of 38 kDa, and two complexes of approximately 34–42 kDa were detected in the hippocampal tissue extract. Since the molecular weights of the TNF-mRNA-binding proteins in the extracts were similar to those of neuronal ELAV-like proteins, we examined the possibility that the TNF-α mRNA-binding proteins in neurons might be neuronal ELAV-like proteins. Anti-neuronal nuclear antibodies, which specifically react with ELAV-like proteins, were incubated with the extract and radiolabeled TNF-mRNA in binding buffer, and resolved on non-denaturing

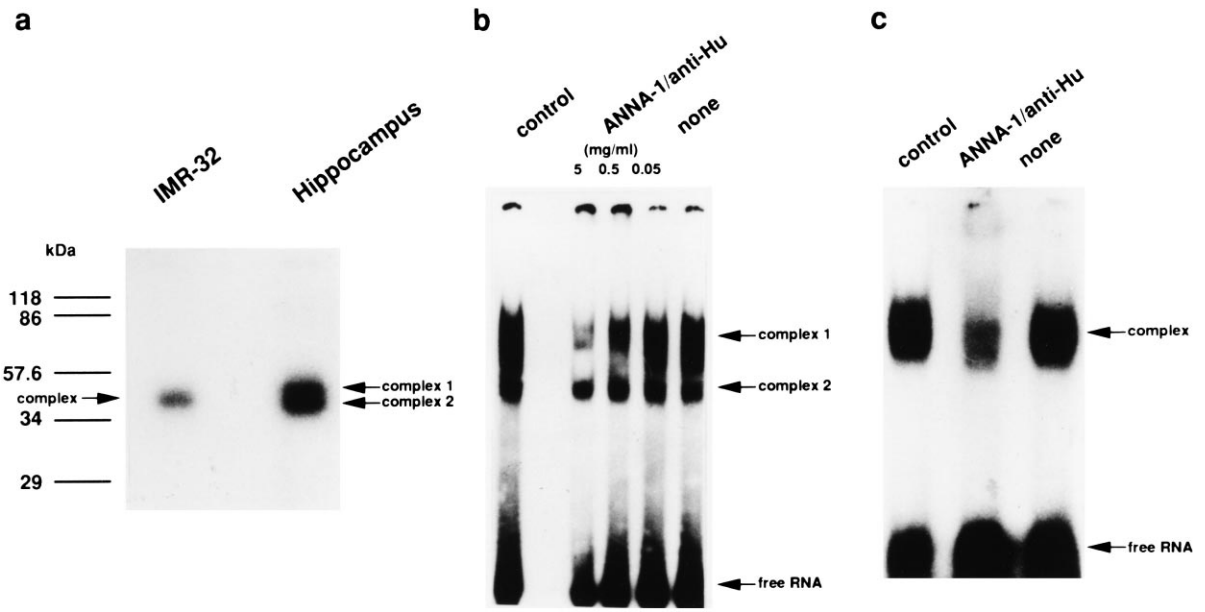

Fig. 1. (a) UV cross-linking analysis of extracts (10 μg) from IMR-32 neuroblastoma line cells and human hippocampus tissue with the radiolabeled 30-mer within the AU-rich sequence in the 3′-UTR of TNF-α mRNA. (b, c) Effects of antibodies against human ELAV-like proteins (ANNA-1/anti-Hu antibodies) on RNA binding of extracts from the human hippocampus tissue (b) and IMR-32 neuroblastoma line cells (c). Control, IgG (0.5 mg/ml) from serum of a healthy individual; ANNA-1/anti-Hu; IgG (0.05 to 5 mg/ml in (b) and 0.5 mg/ml in (c)) from serum of a patient with paraneoplastic neurologic syndromes; none, no IgG was added in the reaction of the extracts and a radiolabeled 30-mer within the AU-rich sequence in the 3′-UTR of TNF-α mRNA.

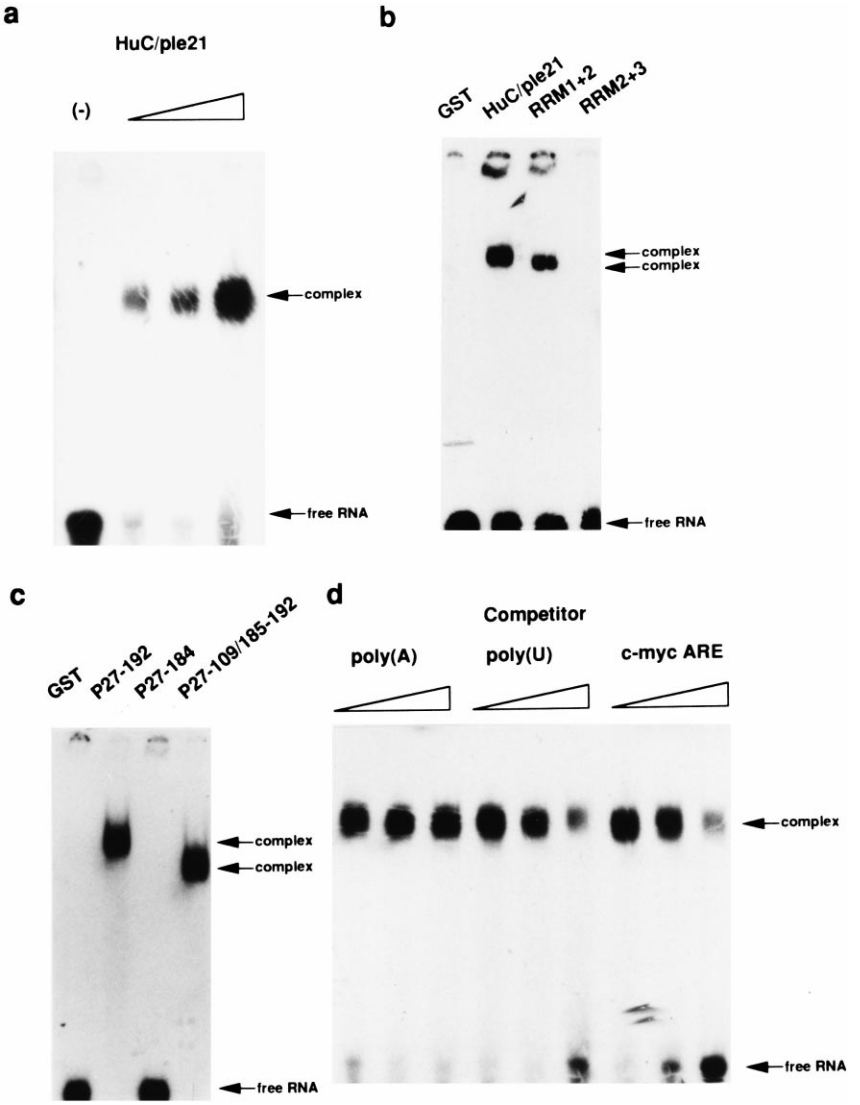

Fig. 2. Binding of the HuC/ple21 recombinant protein (0.02 to 2.0 μg/reaction) (a), two tandem RNA recognition motifs (RRM) of HuC/ple21 (0.2 μg/ml) (b), and deletion mutants of HuC/ple21 RRM1+2 (c) to the 30-mer within AU-rich sequence in the 3′-UTR of TNF-α mRNA. (−), no recombinant protein in the reaction. Slope in (a) indicates increasing concentrations of HuC/ple21. (d) Competitive analysis of binding of HuC/ple21 to the 30-mer within ARE in the 3′-UTR of TNF-α mRNA by increasing concentrations (0.045 to 4.5 mM) of unlabeled poly(U) and poly(A) ribohomopolymers, and unlabeled 30-mer within ARE in the 3′-UTR of c-myc mRNA. Slopes indicate increasing concentrations of each competitor.

gels. In the gel-shift assay with the hippocampal tissue extract, two protein-RNA complexes were detected (Fig. 1b). The addition of antibodies decreased the intensity of the autoradiographic signals of the slower migrating complex in a dose-dependent manner (0.05 to 5 mg/ml), while the addition of control IgG (0.5 mg/ml) did not affect the intensity. The intensity of the faster migrating complex was unchanged by the addition of antibodies. With cell extract of neuroblastoma cell line IMR-32, a single protein-RNA complex was detected in the gel-shift assay. The addition of antibodies (0.5 mg/ml) reduced the intensity of the complex's autoradiographic signal (Fig. 1c). These results suggest that neuronal ELAV-like proteins might bind to TNF-α mRNA.

3.2. *The binding of neuronal ELAV-like proteins to TNF-α mRNA*

We then examined the binding of neuronal ELAV-like proteins to TNF-α mRNA using a recombinant protein, HuC/ple21, which we previously cloned from a human hippocampal cDNA library [12], as the neuronal ELAV-like protein. The gel-shift assay using the recombinant protein and TNF-α mRNA revealed that this neuronal protein formed a complex with TNF-mRNA (Fig. 2a). The gel-shift assay using RNA recognition domain (RRM) deletion mutants indicated that the first two tandem RRMs bound to the oligoribonucleotide, but not the last two, as previously demonstrated with mRNA for interleukin-3 (IL-3), c-myc, and c-fos (Fig. 2b). Furthermore, a deletion in the first two RRM peptides showed that HuC/ple21 amino acids 185–192 were important for binding to TNF-α mRNA, as was observed in the binding of the protein to mRNA for c-myc and IL-3 (Fig. 2c). Competitive binding of HuC/ple21-TNF-α mRNA with poly(U) and poly(A) homopolymers, and the 30-mer oligoribonucleotide corresponding to the ARE in the 3′-UTR of c-myc

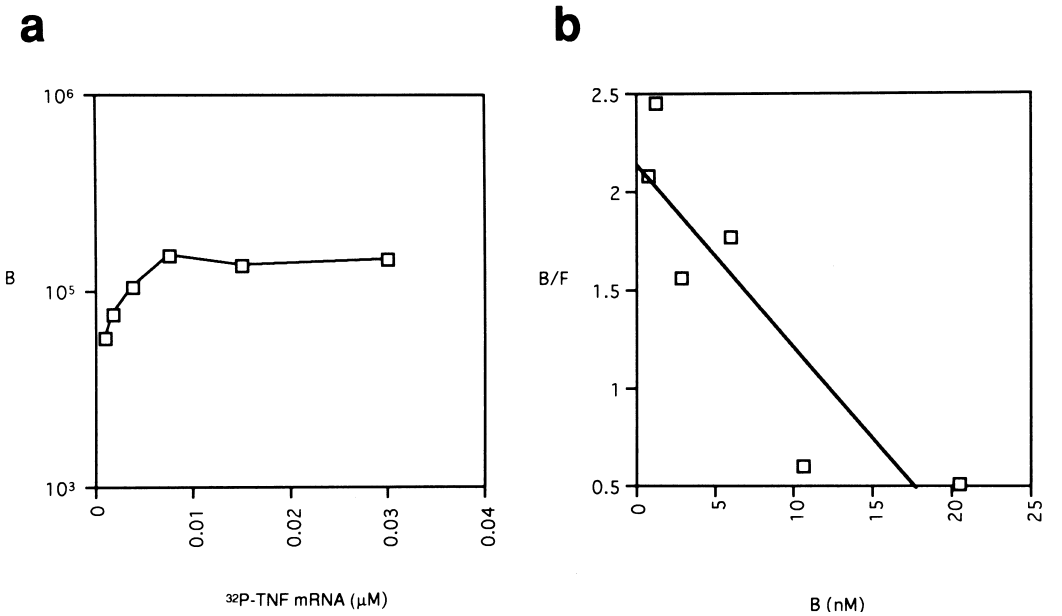

Fig. 3. (a) Binding curve of TNF-α mRNA to HuC/ple21. Recombinant HuC/ple21 protein and increasing concentrations of ^{32}P-labeled TNF-α mRNA were incubated for 10 min at 37°C with excess amount of unlabeled TNF-α mRNA (1.5 μM). B, ^{32}P-labeled TNF-α mRNA specifically bound. (b) Scatchard analysis of the binding of TNF-α mRNA to HuC/ple21. Bound to free TNF-α mRNA (B/F) is plotted against bound ^{32}P-labeled TNF-α mRNA (B).

mRNA showed that the poly(U) homopolymer and c-myc mRNA efficiently competed with TNF-α mRNA to bind to HuC/ple21, but the poly(A) homopolymer did not (Fig. 2d). The binding plots indicated that TNF-α mRNA bound to HuC/ple21 with affinity in a saturable manner. A Scatchard analysis showed that TNF-α mRNA bound to the neuronal ELAV-like protein with an apparent dissociation constant (K_d) of 10.8 nM (Fig. 3).

3.3. *Effects of cytokines that regulate TNF-α on neuronal ELAV-like proteins*

It has been demonstrated that INF-γ and LPS stimulate TNF-α production in monocytes, macrophages, and IMR-32 neuroblastoma cells [11,16–18]. In contrast, IL-4 suppresses TNF-α production [19–22]. We investigated whether these cytokines affected the expression of neuronal ELAV-like proteins in IMR-32 neuroblastoma cells. Immunoblot analysis showed that treatment with INF-γ and LPS did not alter the expression of ELAV-like proteins. On the other hand, the expression of ELAV-like proteins decreased in the neuroblastoma cells treated with IL-4 for 2 h (Fig. 4a). Less ELAV-like protein was induced with a longer treatment of IMR-32 cells with IL-4 (Fig. 4b).

4. Discussion

In this study, we described that cytosolic proteins contained in human hippocampal tissue and neuroblastoma line cell extract bind to a 30-mer oligoribonucleotide within the ARE in the 3′-UTR of TNF-α mRNA, and that the proteins are neuronal ELAV-like proteins. The production of cytokine is regulated both at the transcriptional and post-transcriptional

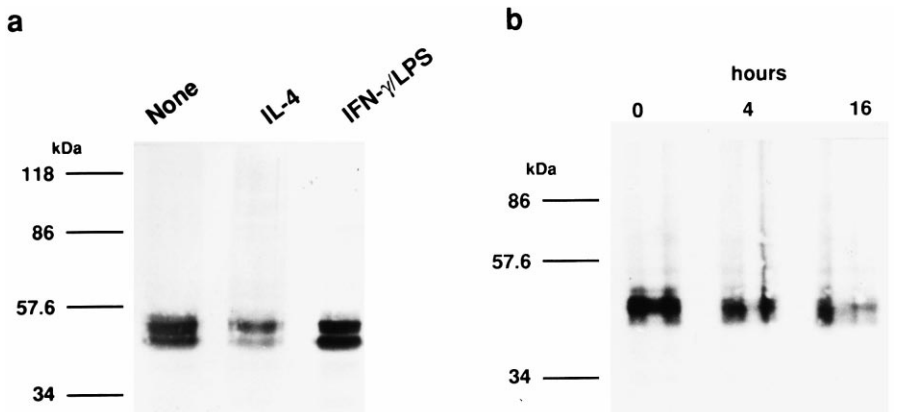

Fig. 4. (a) Effect of IL-4 or INF-γ/LPS on the expression of human neuronal ELAV-like proteins in IMR-32 neuroblastoma line cells. Cytosolic lysates were isolated from cells treated with IL-4 (10 ng/ml) or INF-γ/LPS (10 ng/ml/1 μg/ml) for 2 h and were analyzed by immunoblotting analysis with ANNA-1/anti-Hu antibodies. (b) Effect of IL-4 on the expression of human neuronal ELAV-like proteins. Cytosolic lysates were isolated from cells treated with IL-4 (10 ng/ml) for the times indicated and analyzed by immunoblotting analysis with ANNA-1/anti-Hu antibodies.

levels. Post-transcriptional control of cytokine expression is conferred by sequences in the 3′-UTR of its mRNA. In many instances, the instability of the mRNA has been mapped to the ARE in the 3′-UTR of TNF-α mRNA. TNF-α mRNA contains several AREs that can influence mRNA stability. The binding of cytosolic and nuclear proteins to the AREs of the mRNA controls post-transcriptional regulation and the binding can alter mRNA stability. Several TNF-α mRNA-binding proteins have been described. Bohjanen et al. described how two cytoplasmic proteins, 34 kDa AU-A and 45 kDa AU-B, in εξτραχτ of the human Jurkat T-cell line bound to the AU-rich sequence of TNF-α mRNA [23]. AU-A is ubiquitous in unstimulated cells, while AU-B is only induced in stimulated T-cells. Hel et al. revealed that at least six macrophage proteins with apparent molecular masses of 48, 52, 54, 81, 101, and 150 kDa bind to TNF-α mRNA [24]. Lewis et al. also described two TNF-α mRNA-binding proteins in monocyte/macrophage cells [25]. Recently, it was demonstrated that tritestoraprolin in macrophages binds to the ARE in the 3′-UTR of TNF-α mRNA, and destabilizes the mRNA [26]. The present study showed that neuron-specific proteins also have the capacity to bind to the ARE in the 3′-UTR of TNF-α mRNA.

TNF-α mRNA oligoribonucleotide contains three AUUUA pentanucleotides and two UUAUUUAUU motifs. Since the binding of HuC/ple21 to TNF-α mRNA competed with the class-1 AU-rich sequence of c-myc mRNA, which contains a single AUUUA, and the formation of the HuC/ple21-TNF-α mRNA complex competed with the poly(U) homopolymer, but not with the poly(A) homopolymer, the HuC/ple21 protein may recognize the clustered uridine stretches in AUUUA, but may not distinguish clustered AUUUA (class-1 ARE) from a single AUUUA (class-2 ARE). It has been shown that neuronal ELAV-like proteins interact with the uridine-rich sequence in the 3′-UTR of mRNA for granulocyte/macrophage colony stimulating factor (GM-CSF), IL-3, c-fos, c-myc, p21 waf, Gap-43, and glucose transporter (GLUT1) [27–31]. These uridine-rich mRNA sequences bind to the first two RRMs of the ELAV-like proteins. Our previous studies confirmed that HuC/ple21 binds to the ARE in the 3′-UTR of IL-3 and c-myc mRNA, and revealed that the entire first RRM and only the octapeptide in the second RRM of HuC/ple21 are necessary and sufficient for binding to IL-3 and c-myc mRNA (Sakai, K. et al., submitted). The present data indicate that the TNF mRNA-binding region of the HuC/ple21 protein appears to be identical to IL-3 and c-myc mRNA-binding regions.

The biological function of HuC/ple21 in TNF-α is not known. Other ELAV-like proteins play roles in post-transcriptional regulation. Peng et al. demonstrated that elevation of HuR ubiquitously expressed ELAV-like proteins in the cytoplasm, although ectopic overexpression of exogenous HuR specifically inhibits the c-fos ARE-directed mRNA decay [32]. The overexpression of Hel-N1, another human neuronal ELAV-like protein, enhances the cytoplasmic expression of GLUT1 mRNA [29]. This evidence suggests that HuC/ple21 might play a regulatory role in TNF-α production. INF-γ and LPS stimulate TNF-α synthesis [11], while IL-4 inhibits it [19,20]. The mechanism by which IL-4 inhibits LPS-induced TNF-α production in monocyte/macrophages is still controversial. IL-4 inhibited the accumulation of TNF-α mRNA in human monocytes by enhancing mRNA degradation [21]. Other reports indicated that IL-4 did not alter TNF-a mRNA accumulation [33]. In unstimulated macrophages, TNF-α production was repressed and the AU-rich sequence in the 3′-UTR of TNF-α mRNA mediated this translational repression. Mijatovic et al. described how IL-4 induced translational repression of TNF-α mRNA through the intermediate ARE in the 3′-UTR of TNF-α mRNA in a macrophage/monocyte cell line [22]. The present study indicated that in vitro treatment of IMR-32 cells with IL-4 decreased the expression of the neuronal ELAV-like proteins. This result suggests the possibility that IL-4 might exert its inhibitory action on TNF-α mRNA by altering the expression of neuronal ELAV-like proteins in neurons and neuroblastoma cells. Further experiments to examine this possibility are under way.

Analyzing the mechanisms for the interaction between neuronal ELAV-like proteins and TNF-α should be important to understand the pathophysiological roles of TNF-α in the brain, and the regulation of neuronal ELAV-like protein expression may be useful to control the pathological CNS conditions that are related to TNF-α.

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